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(54) Title: A REPORTER PROTEIN CONSTRUCT, PARTLY COMPRISING GPF

(57) Abstract: The present invention refers to a reporter protein construct comprising a reporter part at the carboxy end and a ubiquitin part (Ub) at the amino end, characterised by that it further comprises a linker peptide between the reporter part and the ubiquitin, whereby the linker comprises at least one Lys-residue, as well as a method for measuring activity of the ubiquitin/proteasome pathway in living cells.

A reporter protein construct, partly comprising GFP.

Technical field

The invention refers to a reporter protein construct, partly comprising GFP, for quantification of ubiquitin/proteasome-dependent proteolysis in living cells, and a method for the detection and screening of accumulated reporter constructs.

General background

Ubiquitin/proteasome-dependent proteolysis has been implicated in the degradation of proteins that control vital processes such as cell cycle progression, signal transduction, differentiation and apoptosis[1]. It also participates in the clearance of misfolded and damaged proteins[2,3] and in the generation of peptides for MHC class I restricted antigen presentation[4]. These diverse roles in proteolysis place the ubiquitin/proteasome pathway at the core of pathologic processes such as inflammation, autoimmunity, neurodegenerative diseases and cancer (reviewed in 5), and motivates the large efforts devoted to elucidate its mechanism of action and identification of compounds that may allow modulation in living cells[6,7]. Indeed, proteasome inhibitors have been proposed as antitumor agents[8,9] and the cancer drugs epoxomicin and eponemycin interact specifically with the proteasome[10,11].

The proteasome is a multicatalytic multisubunit protease composed of a proteolytic core, the 20S proteasome, sandwiched between 19S/PA700 or 11S/PA28 regulatory complexes (reviewed in 12). The 19S regulator is involved in the ATP-dependent recognition and unfolding of proteins that are "marked" for degradation by covalent attachment of multiple ubiquitin molecules (reviewed in 1). The specificity of this process is safeguarded by signals that identify the substrates for recognition and ubiquitin-dependent degradation[13]. The 20S core consists of two heptameric outer α-rings and two inner β-rings that form a barrel-like structure. Only three of the β subunits, β5 (X), β2 (Z), and β1 (Y), are amino-terminal threonine hydrolases that execute proteolysis with chymotrypsin-like, trypsin-like and post glutamyl peptide

hydrolysing (PGPH) specificity, respectively[14]. The latter activity was recently defined as caspase-like due to its preferential cleavage after aspartic residues[15].

Analysis of yeast mutants and chemical inhibitors have revealed an unexpected complexity of proteasome function with contributions from individual catalytic subunits, cooperative interactions between subunits[16] and allosteric effects triggered by binding of the substrates or cleavage events[15]. Most the studies testing the activity of the ubiquitin/proteasome pathway rely on the use of purified proteasomes and fluorogenic substrates that diffuse into the enzymatic chamber and are cleaved in an ATP-independent manner. This experimental setup and the available technology for measuring the activity of the proteasome do not reproduce the complex interactions leading to ATP-dependent degradation of ubiquitinated proteins and do not assess the influence of vital parameters, such as bioavailability and cell permeability, that may affect the therapeutic value of proteasome inhibitors.

Thus, there is a need for new systems and methods for monitoring proteasomal activity, avoiding the drawbacks mentioned above.

Some references in the field are WO99/11774, which discloses a polypeptide-reporter molecule that may comprise a ubiquitin part, a GFP-part and a "coiled coil"-domain, as well as a method in which this molecule is used, and WO98/57978, which refers to proteasome purification and discloses a fusion protein that may comprise a ubiquitin part and a signal producing part.

Furthermore, US-A-5132213 discloses a gene construct comprising a ubiquitin part, a reporter part such as β-galactosidase, as well as an interjacent part. The interjacent part comprises lysine, which is regarded to be important for destabilisation. Moreover a method for producing a fusion protein of the construct above is disclosed. This construct is adapted for use in yeast cells.

None of the above mentioned documents describe reporter systems that can be used for monitoring proteasomal degradation in living cells. Application WO99/11774

refers to a model protein which can be used for identification of proteins that interact specifically with proteins that undergo posttranslational modifications, such as ubiquitination. Application WO98/57987 is based on fusion of a reporter with different type of domains, often referred to as 'ubiquitin-like domain'. The inventors exploited the unique properties of ubiquitin-like domains for the purification of proteasomes from cell lysates. As outlined in WO98/57987, this property is unique for ubiquitin-like domains and deviates in this respect, both structurally and functionally, from the introduction of a poly-ubiquitination signal in GFP as enclosed in the present document. The third document, US-A-5132213, presents a method for using ubiquitin fusions to regulate protein stability. This study is solely based on studies in yeast and the proposed reporters were later shown to be ineffective in cells of higher eukaryotes. Moreover, the method of US-A-5132213 exploits as readout the enzymatic activity of a protein and lacks therefore the unique feature of the GFP reporter, namely the possibility to monitor the ubiquitin/proteasome pathway in living cells.

Summary of the invention

By the construction of a reporter construct for proteasomal activity comprising GFP with, for example, a destabilising N-terminus, the inventors have succeeded in providing a system, in which proteasomal activity in living cells can be measured through the accumulation of the reporter protein. While the reporter protein preferably is GFP, the accumulation can easily be quantified.

Specifically, the invention refers to a construct encoding a reporter protein comprising a reporter part at the carboxy-terminal and an amino-terminal part comprising ubiquitin (Ub) or a functional analogue. The reporter is characterised by that it further comprises a linker peptide between the reporter part and the ubiquitin, whereby the linker comprises at least one Lys-residue, and whereby position 3 of the linker is occupied by a Lys-residue.

Moreover, the invention concerns a method for the measurement of the activity of the ubiquitin/proteasome pathway, characterised by that the method is performed in living cells, and that it comprises the steps of (1) expressing a reporter construct of any of claims 1-9 into the cell, (2) optionally adding substances to inhibit the ubiquitin/proteasome dependent proteolysis, and (3) measuring the presence of accumulated reporter protein.

Such a method could for example be used for high-throughput screening of substances, which selectively modify proteolysis in-vivo.

Accordingly, the reporter can be used as a measure of the proteasome inhibiting potential of added substances, or to study whether a cell in it self has an defective proteasome function.

To summarise, the idea of the invention is to use a modified GFP-reporter protein to measure the presence of ubiquitin/proteasome-activity in vivo, whereby the reporter protein accumulates in a cell with defective ubiquitin-proteasome dependent proteolysis or in a cell when substances inhibiting the proteolytic pathway are used.

Advantages

Some of the advantages of the present invention is that it can be used to measure/study proteasomal activity in living cells, it provides a very sensitive detection system, it can be used to measure partial degradation and quantify destabilisation, as well as to screen for other (earlier) steps of the ubiquitin/proteasome pathway. Moreover, it can be used to distinguish between qualitative modification of the pathway and induction that leads to apoptosis. Furthermore, the construct of the invention is specifically aimed for higher eukaryotic cells, specifically mammalian cells.

Definitions

A reporter protein construct here refers to a molecule having the ability to be detected when it accumulates in a cell, such as a molecule having a GFP (green fluorescent protein)- part, and which thus is detectable through fluorescent techniques.

The reporter part is the part of the fusion protein having the ability to give rise to detectable signals. Reporters such as enzymes, for example β-galactosidase; bioluminiscence, luciferase, or fluorescent proteins, such as GFP, EGFP; or modified variants of these reporter proteins, can be used. Preferably, GFP is used.

The ubiquitin part refers to the part of the reporter protein construct comprising a ubiquitin molecule or any functional analogue, such as a ubiquitin having one or more mutations.

A functional analogue refers to any proteins or protein domains that once inserted at the amino terminus of a substance will target this substance for degradation by the proteasome.

Detailed description of the invention

Because of its stability and easily detectable green fluorescence, GFP is a widely used reporter in studies of gene expression and protein localisation[26]. The inventors have exploited these properties to generate the first reporter that allows in vivo quantification of ubiquitin-proteasome-dependent proteolysis in mammalian cells. The inventors show that GFP can be directly targeted to the ubiquitin-proteasome pathway by insertion of N-end rule and UFD degradation signals.

In previous studies the N-end rule and UFD targeting signals were shown to generate artificial substrates with variable half-lives in yeast cells, depending on the identity of the N-end residue[17] or efficiency of cleavage of the N-terminal ubiquitin by ubiquitin hydrolases[18]. The efficacy of these targeting signals depended on the presence of a specific linker sequence, e^K, directly adjacent of the ubiquitin moiety.

However, these constructs were found to be stable in cells of higher eukaryotes. Only insertion of a linker derived from a viral protein resulted in destabilization of the substrate although even these optimized substrates remained more stable than the e^K-linker counterparts in yeast (ref). Difficulties in generating destabilized ubiquitin fusions in higher eukaryotes were further highlighted in several subsequent studies in which absence of stabilization was observed. For example, a Ub-GFP fusion had a half life of up to 4 hr in a slime mould, whereas the half- life was only few minutes in yeast cells (ref). In a second study it was shown that a Ub-GFP fusion was not targeted for proteasomal degradation in extracts of mammalian cells and remained fully stable (ref). Thus while destabilization can be obtained in yeast using the ubiquitin fusions methodology, the same technique is more demanding when applied in cells of higher eukaryotes for reasons not fully understood.

The inventors of the present invention developed a set of Ub-GFP fusions provided with a specific linker sequence that were efficiently destabilized in mammalian cells. This was confirmed in experiments performed by the inventors by the inverse correlation between the steady state expression of the reporters and the level of accumulation induced by treatment with inhibitors of the proteasome. Thus, while Ub^{G76V}-GFP was not detected in stable transfectants, supporting the notion that proteins carrying this UFD targeting signal may be cotranslationally degraded[18], blockade of the ubiquitin-proteasome pathway resulted in more then 100 fold increase in fluorescence intensity. Positioning of the putative ubiquitination sites in Lys 3 and 17 is likely to be instrumental for this effect, as suggested by the effect of Lys 3 on the degradation of N-end rule-tagged β-galactosidase in yeast cells[27]. The low background fluorescence and efficient accumulation induced by proteasome inhibitors provide a sensitive tool for quantification in individual live cells, without need for time-consuming biochemical assays.

Defects of the ubiquitin-proteasome pathway leading to abnormal degradation of cell cycle regulators and pro-apototic proteins were shown to correlate with uncontrolled cell growth suggesting that proteasome inhibitors may arrest or retard

cancer progression[8-10]. Screening for such potential anticancer drugs has relied on in vitro enzymatic assays using cell homogenates or purified proteasomes, or on cumbersome cytotoxicity tests[9]. The inventors show that the GFP reporters provide an accurate and convenient method to assess biologically significant levels of inhibition. Comparison of peptide hydrolysis and Ub^{G76V}-GFP accumulation in lysates of stably transfected cells demonstrated that the enzymatic activity of the proteasome can be substantially curtailed without detectable accumulation of the reporter. This redundancy is likely to be required during stress responses; when the amount of proteasome substrates, mainly in the form of misfolded proteins, is rapidly elevated[3,28]. Indeed, reduction of proteasomal activity resulted in delayed clearance of the reporter when the amount of substrate was elevated by treatment with reversible inhibitors. More importantly, the correlation between stabilisation of Ub^{G76V}-GFP and induction of apoptosis demonstrates that the reporter truthfully reflects the fate of short-lived proteins, like cyclins and p53, whose inactivation is tightly regulated by ubiquitin-dependent proteolysis[29]. It is noteworthy that, while the cytotoxic effect of proteasome inhibitors requires between 24 to 48 h, a significant accumulation of the GFP reporter was already evident after 2 h.

The involvement of the ubiquitin-proteasome pathway in the production of peptides for MHC class I restricted presentation (reviewed in 4), underlies its potential role in the regulation of immune responses, and the possibility to exploit specific modulators for the treatment of autoimmune diseases, chronic immunopathologies, or diseases caused by transplantation reactions. While the sequence of events leading to protein degradation remains largely unknown, it is clear that the specificity of the catalytic β-subunits influences the characteristics of the antigenic products[30]. Thus, selective inhibition of the chymotrypsin-like activity of the 20S proteasome by the HIV protease inhibitor ritonavir correlated with inhibition of antiviral responses in choriomeningitis virus infected mice and markedly decreased production of certain MHC class I restricted T cell epitopes[24]. A critical requirement for the use of proteasome inhibitors as immunomodulators is that individual enzymatic activities should be blocked without significant effect on proteolysis. Studies with yeast mutants have demonstrated that each of the catalytic subunits of

the proteasome can be eliminated without loss of viability while elimination of more than one specificity is lethal[14]. Proteasomes lacking one of the activities were shown to generate a new set of peptides from protein substrate in vitro[31]. The inventors results suggest that a similar situation may be achieved in mammalian cells. Three proteasome inhibitors, NP-LLG-VS, YL_3-VS and ritonavir were unable to prevent the degradation of Ub^{G76V}-GFP over a wide range of concentrations, while accumulation of the reporter was induced by mixtures of the inhibitors. The cleavage of fluorogenic substrates by proteasomes isolated from treated cells demonstrates that these substances have largely overlapping effects in vivo. Thus, more specific inhibitors will be required to achieve selective changes in the composition of peptides produced by the proteasome. Furthermore, while all cells carrying the Ub^{G76V}-GFP reporter responded to a combination of ritonavir and YL_3-VS, only approximately half of the cells responded to a combination of YL_3-VS with N-LLG-VS. It appears, therefore, that the sensitivity to these inhibitors is dependent on parameters, such as cell permeability and/or expression of the relevant target structure, that are unevenly distributed in a cell population or in different cell types. This possibility is substantiated by the findings of Schmidtke et al. that 50 μM ritonavir induced G1/S arrest in human lymphoblastoid cells[25] while a higher concentration did not affect the cell cycle distribution of Hela cells in the inventors experiments (not shown).

In conclusion, the inventors findings identify N-end rule and UFD-tagged GFPs as a new powerful tool for high throughput screening of inhibitors that may interfere with multiple steps of the ubiquitin-proteasome pathway; including ubiquitin tagging, unfolding and proteolysis. The pharmacodynamics of the inhibitors may be readily evaluated by treating stable transfectants in 96 well plates where the efficiency of proteolysis can be analysed by monitoring changes of fluorescence intensity. The possibility to directly correlate ubiquitin/proteasome-dependent proteolysis with toxicity may facilitate the determination of therapeutic index and dose escalation in clinical trials. An important aspect of the invention is the possibility to use the GFP-reporters for discriminating between inhibitors with cytostatic or immunomodulating activities based on whether they do or do not block

ubiquitin-proteasome dependent proteolysis. In combination with the well established in vitro enzymatic assays, this reporter system may help to achieve a more complete understanding of the distinct effects of proteasome inhibitors on protein degradation.

Accordingly, one main object of the invention is a reporter molecule having one reporter part, preferably GFP, and one of either two destabilising tags, targeting the molecule to a degradation pathway.

Another object of the invention is a linker between the reporter part and the Ub-part, whereby the linker functions as a separator that will enhance degradation.

Definition of the linker sequence between ubiquitin and GFP:

A sequence that once inserted at the N-terminus of GFP places a Lys residue in position 15, 16 or 17 of the chimeric protein. The linker sequence should contain an N-end rule destabilizing amino acid at position 1, most preferably an Arg, Glu, Gln, Trp, Phe, Lys, His, Tyr, or Asp residue; less preferable an Ala, Ser, Leu, Thr, Val, Ile, Gly or Cys residue. Said sequence should also contain a Lys residue at position 3. Other Lys residues may be, but are not necessarily, included in any of the positions between the Lys residue in position 3 and the Lys residue in position 15, 16, or 17.

Sequence 1

$N_1X_2K_3X_{8-10}$

N_1 = N-end rule residue in position 1
X_2 = any residue in position 2
K_3 = Lys residue in position 3
X_b = polypeptide of 8, 9, or 10 amino acids

Sequence 2

$N_1X_2K_3X_{8-10}K_b$

N_1 = N-end rule residue in position 1
X_2 = any residue in position 2
K_3 = Lys residue in position 3
X_{8-10} = polypeptide of 8, 9, or 10 amino acids
K_b = Lys residue in position 13, 14 or 15

Sequence 3
$N_1X_2K_3X_{11-13}(K)$

N_1 = N-end rule residue in position 1
X_2 = any residue in position 2
K_3 = Lys residue in position 3
$X_{11-13}(K)$ = polypeptide of 11, 12, or 13 amino acids with additional Lys residue(s)

Sequence 4
$N_1X_2K_3X_{11-13}(K)K_c$

N_1 = N-end rule residue in position 1
X_2 = any residue in position 2
K_3 = Lys residue in position 3
$X_{11-13}(K)$ = polypeptide of 11, 12, or 13 amino acids with additional Lys residue(s)
K_c = Lys residue in position 15, 16 or 17

The length of the linker peptide is normally in the interval from 3 to 30 amino acids, preferably 11-17 amino acids.

Still another object of the invention is a DNA construct, encoding the above mentioned peptides and proteins of the invention.

One further object of the invention is a method for the measurement of proteasomal activity characterised by that the method is performed in living cells, and that it comprises the steps of (1) introducing a reporter construct into a cell, (2) optionally adding substances to inhibit the proteasomal activity, and (3) measuring the presence of accumulated reporter construct, whereby the accumulation of the reporter construct is used as a measure of ubiquitin-proteasome dependent proteolysis.

The introduction of the reporter construct into the cell can be performed by use of a transfer vector, such as a recombinant vaccinia virus vector, adenovirus vector, retrovirus vector, Semliki forest virus vector, and any other type of viral and nonviral vector, comprising a liposome vector.

Substances inhibiting the proteasomal activity are for example:
- vinyl sulfone-based inhibiotrs such as carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone (Z-L3-VS), nitrophenol leucyl-leucyl-glycine vinyl sulfone (NP-LLG-VS); tyrocyl-leucyl-leucyl-leucine vinyl sulfone (YL3-VS)[22,23].
- peptide aldehyde inhibitors such as carboxybenzyl-leucyl-leucyl-leucinal (MG132)[21]
- lactacystin or its active derivative clasto-lactacystin β-lactone
- peptide boronic acids such as PS-341[8]
- epoxomicin[11]
- eponemycin[10]
- peptide α',β'-epoxyketones[36]

The presence of accumulated reporter molecule can for instance be measured by FACS-analysis or any other fluorescent-based detection method.

A full block of the pathway will lead to apoptosis. The reporter system can tell whether a cell will die in a few days or not. Further, the reporter system can distinguish between modification of pathway and cell death. Some of the major applications of the method are described below.

Application of Ub-X-GFP reporter system

1) Screening for inhibitors of proteolytic activity of proteasome (block ubiquitin-proteasome pathway).

Blockage of the ubiquitin-proteasome pathway leads to induction of apoptosis in proliferating cells. Reporter-expressing cells become fluorescent upon abrogation of proteasomal degradation. Therefore reporter-expressing cells can be used in high throughput screens for the identification of compounds that block proteasomal degradation in living cells. To the inventors knowledge there is no such screen available in living cells. The commonly applied assays rely on the in vitro cleavage of different fluorogenic substrates for the three proteolytic activities of the proteasome. The inventors have demonstrated (fig 4B and 5) that information regarding the in vitro inhibitory activity of the proteasome inhibitors is insufficient to conclude whether the compound will block the proteasomal degradation in vivo.

Assay: One aspect of the invention is to incubate reporter-expressing cells in a multi-well plate with different compounds and monitor the fluorescence intensity of the cells by flow cytometry or fluorimetry.

This can be used in order to identify compounds that block the ubqiuitin-proteasome pathway by inhibition of the proteolytic activity, and thus have anti-tumor activity, anti-inflammatory activity and anti-angiogenic activity.

2) Screening for potential immunomodulators in the form of partial inhibitors of proteolytic activity of the proteasome (compounds that modify proteolytic activity without blocking ubiquitin-proteasome pathway).

Proteasomes are involved in the generation of peptides, which in complex with MHC class I can provoke an attack by cytotoxic T cells. This mechanism is crucial in the elimination of viruses or other intracellular pathogens. In addition this

reaction may contribute to the elimination of malignant cells. In some autoimmunologic disorders this response is disturbed and reacted against normal cells. Others have demonstrated in yeast that inactivation of one of the three proteolytic activities of the proteasome leads to generation of a different set of peptides without affecting the overall ubiquitin-proteasome pathway. Recently it has been demonstrated that an HIV1 protease inhibitor, which cross reacts with the proteasome and changes the proteolytic activity of the proteasome, modifies the response to viral infection in mice. The inventors demonstrate in this study that compounds that do not block the ubiquitin-proteasome on their own can do this in combination with another partial inhibitor. Thus, potential immunomodulators can be identified by screening a library of compounds in combination with another inhibitor, which does not block this proteolytic pathway on its own. This synergistic effect of inactive inhibitors has not been shown before nor has anyone proposed to screen for immunomodulators with library of compounds in the presence of another partial inhibitor.

Another aspect of the invention is to incubate reporter-expressing cells with an inhibitor, which does not or poorly blocks the ubiquitin-proteasome pathway (for example YLLL-VS) in combination with a library of compounds. Cells will become fluorescent if the mixture blocks the ubiquitin-proteasome pathway, which can be monitored as mentioned in 1. Both full inhibitors (as mentioned in 1) and partial inhibitors (potential immunomodulators) will be identified.

Partial inhibitors can also be used to abrogate an unwanted immune response, such as autoimmune disorders or uncontrolled inflammatory response. The same inhibitors may also be helpful to provoke an immune response (for example in cancer patients). As a third possibility the inhibitors can be used to modify the quality of the peptide expressed by the target cell, so that the cell is recognised by T-cells specific for TAP-independent epitopes.

3) Screening for chemical agents/polypeptide sequences that, by inhibition in cis or trans, block the ubiquitin-proteasome pathway of a pre-proteolytic step, like ubiquitination or transport of ubiquitinated proteins to the proteasome.

The presently available inhibitors of the ubiquitin-proteasome pathway, including those presently tested in trials as anticancer agents, focus on the proteolytic activity of the proteasome. With the in vitro assays (see 1) inhibitors of proteolytic activity are relatively easy to find. An important drawback of the usage of inhibitors of proteolytic activity is that each of these inhibitors inhibits also other cellular proteases. A much more specific inhibition of the ubiquitin-proteasome pathway can be accomplished by blocking a step in the pathway before the actual degradation of the proteasome. For example, the ubiquitination step could be attacked. Possible candidates are ubiquitin hydrolases, which are important for the recycling of ubiquitin, or one of the different enzymes involved in ubiquitin tagging, such as the ubiquitin activase, ubiquitin conjugases or ubiquitin ligases. To the inventors knowledge there is no assay available that allows screening of such compounds.

Still another aspect of the invention is an assay as described in 1. In the assay described in 1 all inhibitors of the ubiquitin-proteasome pathway regardless of the step at which they interfere will be identified.

This can be used for the same applications as in 1. However, this type of compounds are expected to be more specific for the inhibitors of the proteolytic activity and should be helpful if malignant cells become resistant to inhibitors of the proteolytic activity.

4) Destabilized Ub-X-GFPs can be used as transcriptional reporters because of their short half life.

GFP is a very stable protein with an extremely long half-life (> 24 hr). For this reason, GFP is not very well suited as a transcriptional reporter. Leaky expression will result in very high background levels and switching off transcription cannot be

monitored since it will take very long before the GFP is cleared from the cells. Others already anticipated that it would be helpful to have GFP variants with shorter half lives and the biotech company Clontech is selling destabilized GFPs as transcriptional reporters which are targeted for proteasomal degradation by introduction of a PEST sequence. The Ub-X-GFPs are similarly targeted for proteasomal degradation and can be used for this purpose.

As still another aspect of the invention, the reporter molecule can be used as a reporter for transcriptional activation.

In a further aspect of the invention the measurement of proteasomal activity in live cells, e.g. cancer cells, before CTL-based immunotherapy, can be used to investigate antigen processing.

Still further, the invention refers to various kits to be used in the different methods of the invention, whereby the kits comprise the chemicals and biomolecules to be used in the methods.

Short description of the drawings

Figure 1. Targeting of GFP for proteasomal degradation. A) Schematic representation of the Ub-X-GFP chimeras. The amino acid sequence of the ubiquitin GFP linking region are shown and relevant amino acid residues are underlined. The amino acid directly downstream of the ubiquitin moiety (bold) is numbered as the first amino acid. The Lys residues in position 3 and 17 are underlined as potential ubiquitination sites. The Gly residue in position -1 of Ub^{G76V}-GFP is substituted by Val. B) HeLa cells transiently transfected with the indicated Ub-X-GFP plasmid were incubated for 10 h without proteasome inhibitor (untreated), or with 50 μM MG132 or 50 (M Z-L_3-VS. Expression of the GFP chimeras was analysed 16 h post-transfection by Western blot with a polyclonal anti-GFP antibody. Polypeptides with sizes corresponding to the cleaved products and the uncleaved precursors are indicated as X-GFP and Ub-X-GFP, respectively. A band corresponding to the Ub-

P-GFP precursor was detected in the treated cells after long exposure of the blots. An additional product, slightly smaller than X-GFP, was detected in Ub^{G76V}-GFP expressing cells upon treatment with inhibitors (asterisk).

Figure 2. Ub-X-GFP expression monitored by fluorescence intensity. A) Flow cytometric analysis of transiently transfected HeLa cells expressing the Ub-X-GFP chimeras. Fluorescent cells are detected in the upper right quadrant. The mean fluorescence intensity and % positive cells are indicated. B) Low magnification fluorescence micrographs of transiently transfected HeLa cells cultured for 10 h in the absence (upper panel) or presence of 50 μM MG132 (lower panel).

Figure 3. Accumulation of GFP reporter upon treatment with inhibitor. A) Emission spectrum of Ub^{G76V}-GFP-expressing cells incubated without or with 10 μM Z-L_3-VS (excitation wave length = 480 nm). The excitation beam and the GFP emission (wave length = 510 nm) are indicated. B) Low magnification micrographs of Ub^{G76V}-GFP-expressing cells incubated for the indicated times in the presence of 10 μM Z-L_3-VS. C)

Figure 4. Correlation between proteasomal degradation and cell growth. A) Cytosolic fractions were prepared from Ub^{G76V}-GFP expressing cells treated with increasing concentrations of Z-L_3-VS and analysed for chymotrypsin-like activity against the suc-LLVY-MCA substrate (closed circles), and GFP fluorescence (open circles). Relative fluorescence is expressed as fold induction over the background of untreated cells. The % hydrolysis of proteasomes from inhibitor treated samples relative to the untreated control is shown. Hydrolysis due to contaminating proteases was measured by addition of 10 μM Z-L_3-VS and 25 μM YL_3-VS during the assay and was subtracted from each sample. Mean of two samples. B) Transfected HeLa cells were treated for 2 h with 5 μM MG132 in order to accumulate Ub^{G76V}-GFP, washed 4 times and cultured for 1 h to allow diffusion of the inhibitor. After one additional wash, the cells were cultured in the absence (closed circles) or presence of 1 μM (open circles) or 2 μM Z-L_3-VS (closed squares) and fluorescence intensity was determined by flow cytometry at the indicated times. The time required for 50%

clearance of accumulated reporter is indicated. Ub^{G76V}-GFP expressing HeLa cell line was cultured in the absence or presence of 2, 4 or 10 μM Z-L_3-VS. C) The mean fluorescence intensity (open circles) and cell numbers (closed circles) were determined at the indicated times. The relative fluorescence is expressed as fold induction over the background of untreated cells. Mean ± SD of three experiments. D) Cell cycle distribution was determined after treatment for 48 h with inhibitor by propidium iodide staining of ethanol fixed cells. The percentages of apoptotic cells and cells in G2/M are indicated. One representative experiment.

Figure 5. Effect of different inhibitors on degradation of reporter. A) Ub^{G76V}-GFP-expressing HeLa cell line was treated for 15 h with increasing concentration of Z-L_3-VS, YL_3-VS, NP-LLG-VS or ritonavir. The fluorescence intensity per well was measured by fluorometry and is expressed as fold induction over background. Mean ± SD of three experiments. B) Flow cytometric analysis of cells treated for 15 h with the indicated inhibitors and inhibitor combinations. One representative experiment out of three.

Figure 6. Over-expression of a mutant ubiquitin results in trans inhibition of the ubiquitin/proteasome pathway. HeLa cells stably expressing the Ub^{G76V}-GFP reporter were transiently transfected with a plasmid encoding the mutant ubiquitin, Ub^{+1}, or with a control vector encoding FLAG-tagged ubiquitin. Two days posttransfection the cells were fixed and stained with a polyclonal anti-Ub+1 antibody or a monoclonal anti-FLAG antibody. A) Ub^{+1} transfected cells were analyzed using fluorescence microscopy for the expression of the Ub^{+1} protein (left panel) and accumulation of the Ub^{G76V}-GFP reporter (middle panel). The cells were counterstained with Hoechst H332258 (right panel). B) The percentage of Ub^{+1} or FLAG-ubiquitin-expressing cells that showed elevated levels of the Ub^{G76V}-GFP reporter were determined by visual inspection. Mean and SD of one experiment performed in triplicates. One representative experiment out of three.

Figure 7. A viral polypeptide blocks in cis the degradation of the GFP reporters. A) GAr polypeptides of 25 and 239 amino acids were inserted at the C-terminus of

GFP reporters that had been targeted for ubiquitin-proteasome dependent degradation by insertion of N-end rule or UFD degradation signals. B) Lysates of HeLa cells transfected with the Ub-M-GFP, Ub-P-GFP, Ub^{G76V}-GFP and Ub-R-GFP plasmids and their GA25 containing counterparts were analysed in Western blots with a polyclonal anti-GFP antibody. The transfected cells were preincubated for 10 h without (-) or with (+) 10 μM of the proteasome inhibitor Z-L_3-VS. The precursor polypeptides, Ub-X-GFP and Ub-X-GFP/GA25, and the cleaved products, X-GFP and X-GFP/GA25, are indicated. Molecular weight markers are indicated on the left. Densitometric analysis of the Western blot is presented in the lower panel. C) Flow cytometric analysis of HeLa cells transfected with GFP, Ub-M-GFP, Ub^{G76V}-GFP and Ub-R-GFP expressing plasmids and their GA25 or GA239 counterparts. Data are expressed as the ratio between the number of fluorescent cells in untreated samples and samples treated with 10 mM Z-L_3-VS. Mean and SD of three experiments. * Significantly different from the corresponding construct without GAr (paired T-test, $P < 0.05$).

Examples

Example 1 – Targeting of GFP for ubiquitin-proteasome dependent proteolysis

N-end rule[19] and ubiquitin-fusion degradation (UFD)[18] signals were used to convert the stable jellyfish GFP into a substrate for ubiquitin/proteasome-dependent proteolysis. Two N-end rule substrates were constructed using Arg (Ub-R-GFP) and Leu (Ub-L-GFP) as destabilising amino acids while two UFD substrates contained a poorly cleavable ubiquitin (Ub-P-GFP) or a mutated uncleavable ubiquitin moiety (Ub^{G76V}-GFP). As control the inventors constructed a Ub-M-GFP chimera that, upon ubiquitin cleavage, does not contain a degradation signal and is therefore expected to be as stable as unmodified GFP. The degradation of N-end rule substrates is dependent on the presence of a Lys residue in position 15 or 17 (ref. 17). Therefore, a random sequence was introduced between the destabilising amino acid and the start codon of GFP, placing the first Lys of GFP in position 17 (Fig. 1A). An

additional Lys was introduced in position 3 of the linker to further increase the efficiency of ubiquitination.

The ubiquitin moiety of the ubiquitin-GFP fusion constructs was obtained by amplifying with the polymerase chain reaction (PCR) the ubiquitin open reading frame from the Ub-Pro-βGal plasmid[32] with the sense primer 5'-GCG GAA TTC ACC ATG CAG ATC TTC GTG AAG ACT-3' (*Eco*RI restriction site, underlined; start codon, bold) and the antisense primers 5'-GC GGG ATC CTG TCG ACC AAG CTT CCC XXX CCC ACC TCT GAG ACG GAG TAC-3' for Ub-X-GFP, or 5'-GC GGG ATC CTG TCG ACC AAG CTT CCC CAC CAC ACC TCT GAG ACG GAG TAC-3' for $Ub^{Gly76Val}$-Val-GFP (*Bam*HI restriction site, underlined; variable codon, bold). The PCR product was cloned, using the *Eco*RI and *Bam*HI restriction sites, in frame with the 5' end of the GFP open reading frame in the EGFP-N1 vector. EGFP-N1 encodes a GFP variant with an optimized codon usage for expression in mammalian cells and a red-shifted excitation spectrum[35]. Human cervical epithelial carcinoma HeLa cells were cultured in Iscove's modified enriched medium supplemented with 10% fetal calf serum (Life Technologies). Transient transfections were performed with Lipofectamine (Life Technologies) in 24 or 12 well plates by incubating the cells for 5 hr in the presence of a transfection mixture containing 0.5 μg plasmid DNA and 1.5 μl lipofectamine/well. Cells were analysed 24 to 48 hours posttransfection. For experiments with proteasome inhibitors, cells were incubated in the presence of 50 μM carboxybenzyl-leucyl-leucyl-leucinal[34] or carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone (Z-L_3VS)[33] or, as a control, with an identical DMSO concentration (0.5 %).

Expression of the GFP chimeras in transiently transfected HeLa cells was investigated by Western blot analysis (Fig. 1B). The transfected cells were harvested 24-48 post-transfection in 100 μl SDS-PAGE loading buffer and lysed by boiling. Total cell lysates of 10^5 HeLa cells were run on 10% SDS-PAGE gels and transferred to Protan BA 85 nitrocellulose filter (Schleicher & Schuell). Filters were blocked for 1 hr in phosphate buffered saline supplemented with 5% skim milk and 0.1% Tween-20 and incubated for 1 hr with rabbit polyclonal anti-GFP serum

(Molecular Probes) diluted 1:1500 in the same buffer. Filters were washed 4 times 5 minutes and incubated for 1 hr with peroxidase-conjugated goat anti-rabbit serum. After 4 washing steps of 10 min, the secondary antibody were visualized with ECL reagents (Amserham) and exposed to Ortho HT-U film (Agfa). The size of Ub-M-GFP, Ub-R-GFP, Ub-L-GFP and Ub-P-GFP, corresponded to the expected size of unmodified GFP, confirming cleavage of the ubiquitin moiety in vivo. Long exposures of the blots revealed the presence of the full length fusion protein in cells expressing Ub-P-GFP, which is in line with the expected slower cleavage of Ub-P-GFP by ubiquitin hydrolases (Fig. 1B, lower panel). Only the uncleaved precursor was detected in cells expressing Ub^{G76V}-GFP.

Consistent differences were observed in the steady state expression of the chimeras (Fig. 1B, untreated samples). The L-GFP and P-GFP polypeptides were always weaker than the stable M-GFP, and the R-GFP and Ub^{G76V}-GFP specific bands were hardly visible. The transfected cells were preincubated in 50 μM of the reversible proteasome inhibitor MG132 (ref. 21) or 50 μM of the irreversible inhibitor Z-L_3-VS (ref. 22) for 10 h. As illustrated in Fig. 1B, both inhibitors induced an increase of GFP inversely proportional to the level of expression in untreated cells. This ranking order of expression is in line with the reported efficiency of the degradation signals in mammalian cells[19,20], suggesting that the chimeras are targeted for proteasomal degradation.

Example 2 – Use of the reporters form measurement of ubiquitin-proteasome dependent proteolysis in living cells

To measure the ubiquitin-proteasome dependent degradation of the reporter in living cells, HeLa cells were transfected as described in Example 1. The transfected cells were preincubated in 50 μM of the reversible proteasome inhibitor MG132 (ref. 21) or 50 μM of the irreversible inhibitor Z-L_3-VS (ref. 22) for 10 h and fluorescence intensity was measured by flow cytometry and fluorescence microscopy. For fluorescence microscopic analysis the cells were grown and transfected on coverslips. The control and transfected cells were fixed in 4% paraformaldehyde in phosphate buffered saline 24-48 hr posttransfection and mounted with 50% glycerol

in PBS. The cells were analyzed with a LEITZ-BMRB fluorescence microscope (Leica) using a bandpass FITC filter setting. Images were captured with a Hamamatsu 4800 cooled CCD camera (Hamamatsu). All images were obtained with equal exposure and processed in parallel in Adobe Photoshop software. For flow cytometry analysis HeLa cells were transiently transfected and harvested by trypsinization 24-48 hr posttransfection. The cells were spin down at 3000 rpm and washed in cold phosphate buffered saline (PBS) once. The cells were analysed directly after collection using a FACsort flow cytometer (Beckton & Dickinson). The data were analysed with Cellquest software. The steady state fluorescence of the transfected cells corresponded to the intensity of the GFP specific band detected in Western blots (Fig. 2A). Treatment with the proteasome inhibitors resulted in accumulation of fluorescence detected by FACS (not shown) and an increase in the number of GFP positive cells detected by and fluorescence microscopy (Fig. 2B). Note that fluorescence measurements detect solely mature GFP while Western blotting detects both mature and immature/unfolded polypeptides.

Example 3 – Production of stable transfectants for quantification of ubiquitin-proteasome dependent proteolysis

A stable transfectant expressing the strongly destabilised Ub^{G76V}-GFP was produced in order to investigate the activity of the ubiquitin/proteasome pathway in living cells. Stable transfectants were generated by selection of cells transfected as described in Example 1 in the presence of 500µg/ ml of G418 (Sigma St. Louis, Missouri). HeLa cells stably transfected with Ub^{G76V}-GFP were incubated with proteasome inhibitor for 10 hrs in a 24 well plate. The cells were harvested by trypsinization and spinned down for 3 minutes in 2000 rpm. The cell pellet was resuspended in 500 µl PBS and the fluorescence was measured with a fluorimeter (LS50B, Perkin Elmer). Excitation wavelength is 480 nm and emission wavelength is 510 nm. The spontaneous fluorescence of the transfectant was only marginally above the autofluorescence of untransfected cells while a characteristic GFP emission peak was detected at 510 nm upon treatment with proteasome inhibitor (Fig. 3A). Fluorimetric analysis and fluorescence microscopy analysis showed that the increase was time- dependent (Fig 3B). In order to test whether the increase of

fluorescence could be easily monitored in multiwell plate assays HeLa cells stably transfected with the Ub^{G76V}-GFP construct were incubated with the proteasome inhibitor Z-L_3-VS for 10 hrs in a 24 well plate. The cells were harvested by trypsinization and transferred to a 96 well plate for immediate analysis of fluorescence with a multilabel plate reader (Victor, Wallac). A dose dependent increase of fluorescence intensity was observed (Fig. 3C). Different fluorimetric assays demonstrated levels of fluorescence reaching 100 to 1000 fold induction, depending on the inhibitor and incubation time (figure 3A, C and 4C). Similar effects were observed upon treatment with the irreversible inhibitor lactacystin (data not shown). The Ub^{G76V}-GFP transfectant was further used to explore the correlation between enzymatic activity of the proteasome and efficiency of proteolysis. Extracts of cells treated with increasing concentration of Z-L_3-VS, were tested in parallel for accumulation of the reporter and cleavage of the fluorogenic substrate Suc-LLVY-AMC. Hydrolysis of the fluorogenic substrates Suc-LLVY-Amc was measured by incubation in 100 µl reaction buffer (50 mM Tris pH 7.5, 5 mM MgCl2, 1 mM DTT) containing 100 µM substrate and 10 µg cytosolic fraction. The cytosolic fraction was prepared by differential centrifugation at 100 000 g (ref 30; supernatant after 1 h 100 000 g) Non-specific hydrolysis was assessed by preincubation for 10 min with 10 µM Z-L3-VS and 25 µM YL3-VS and was found to be less then 10% of the total activity. Specific activity was expressed as the hydrolysis of untreated samples minus the hydrolysis of samples pretreated with inhibitors. GFP fluorescence was monitored in parallel in the cytosolic fractions. In line with the effect of Z-L_3-VS on purified proteasomes, a dose-dependent inhibition of the chymotrypsin-like activity. Degradation of the reporter was also affected in a dose-dependent manner but, surprisingly, blockade of the ubiquitin/proteasome pathway became evident only at 4 µM Z-L_3-VS when the chymotrypsin-like activity was inhibited by more than 80% (Fig. 4A). The inventors reasoned that functional impairment might be visualised by challenging the cells with increased amounts of substrate, a situation that mimics the accumulation of misfolded proteins during stress responses. Transfected HeLa cells were for 2 h with 5 µM MG132 in order to accumulate the reporter, washed 4 times and cultured for 1 h to allow diffusion of the inhibitor. After one additional wash, the cells were cultured in the absence or

presence of different concentrations of Z-L3-VS and fluorescence intensity was determined by flow cytometry at the indicated times. A 7 fold increase of fluorescence was induced by preincubation for 2 h with 5 μM of the reversible inhibitor MG132 and clearance of the reporter was then monitored in medium alone or in the presence of 1 or 2 μM Z-L_3-VS (Fig. 4B). The fluorescence half-life was approximately 2 h in the absence of inhibitor and 3 and 4 h in the presence of 1 and 2 μM Z-L_3-VS, respectively. This dose-dependent delay confirms that the activity of the proteasome is affected also at low concentrations of the inhibitor and shows that the reporter system can be used to monitor suboptimal inhibition of ubqiuitin-proteasome-dependent proteolysis.

Example 4– Use of the reporter to assess induction of apoptosis by inhibition of the ubiquitin-proteasome pathway

The inventors evaluated whether accumulation of the reporter is a predictor of biologically relevant levels of inhibition. Cells cultured in the presence of increasing concentrations of Z-L_3-VS were compared for fluorescence emission, cell cycle distribution and induction of apoptosis. HeLa cells stably expressing the UbG76V-GFP reporter were cultured in the presence of different concentrations of Z-L3-VS. At the different times cell number and viability were assessed by counting in trypan blue. For flow cytometric analysis of the fluorescence UbG76V-GFP expressing HeLa cells were harvested by trypsinization, spun down at 3000 rpm and washed in cold phosphate buffered saline (PBS) once. The cells were analysed directly after collection using a FACsort flow cytometer (Beckton & Dickinson). The data were analysed with Cellquest software. The increase in fluorescence is expressed as induction of the mean fluorescence intensity over background. Cell cycle distribution and the percentage of apoptotic cells were determined with propidium iodide staining. The DNA content and GFP fluorescence were determined in parallel by flow cytometry (Fig. 4C). Failure to accumulate GFP in cells treated with 2 μM Z-L_3-VS correlated with normal G1-G2/M distribution and undisturbed growth. In contrast, the accumulation of GFP was accompanied by arrest in G2/M, an induction of apoptosis and a reduction in cell number. Similar effects were observed on treatment of the parental HeLa cells, excluding the contribution of toxicity due to

GFP accumulation (data not shown). Thus, accumulation of the UbG76V-GFP is predictive for induction of apoptosis induced by inhibition of the ubiquitin-proteasome pathway.

Example 5 – Use of the reporter to identify partial inhibitors of proteasomal activity

The inventors assessed the applicability of the reporters for the identification of the activity of compounds that selectively modify the catalytic function of the proteasome. The inventors chose three recently identified inhibitors: carboxybenzyl-leucyl-leucyl-leucinal (MG132), carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone (Z-L_3-VS), nitrophenol leucyl-leucyl-glycine vinyl sulfone (NP-LLG-VS); tyrocyl-leucyl-leucyl-leucine vinyl sulfone; (YL_3-VS), and the HIV-1 protease inhibitor ritonavir. Each of these inhibitors was shown to preferentially affect the hydrolysis of certain fluorogenic substrates[23-25]. In particular, YL_3-VS preferentially inhibits the trypsin-like activity while NP-LLG-VS inhibits the chymotrypsin- and trypsin-like activities but has virtually no effect on the PGPH activity (23 and Hidde Ploegh, personal communication). Ritonavir inhibits the chymotrypsin-like activity but induces the trypsin-like activity and affects the presentation of certain MHC class I restricted T-cell epitopes[24,25]. HeLa cells stably expressing the reporter were treated with inhibitors and analyzed by fluorescence spectrometry as described in Example 3. The fluorescence is expressed as fold increase of total fluorescence over background. Treatment of Ub^{G76V}-GFP-expressing cells with up to 100 μM YL_3-VS did not induce accumulation of the reporter and a weak effect was observed at concentration of NP-LLG-VS above 50 μM (Fig. 5A). Ritonavir had a very weak effect that reached a plateau at 60 μM. In parallel, the inventors evaluated in the treated cells the activity of each of the enzymatic activities of the proteasome. Hydrolysis of the fluorogenic substrates Suc-LLVY-AMC (chymotrypsin-like), Boc-LRR-AMC (trypsin-like) and Cbz-LLE-βNA (PGPH; Sigma) by 5 μg proteasomes semipurified from treated cells was measured as described in Example 3. The semipurified proteasomes were obtained by resuspending the pellet after 100 000 g centrifugation for 5 h in assay buffer with 50% glycerol (30). The activity of the inhibitors was confirmed by the reduced hydrolysis of fluorogenic substrates by proteasomes isolated from cells treated with YL_3-VS and NP-LLG-VS (Table I).

However, the pattern of inhibition correspond poorly to the reported activity of these substances on purified proteasomes, suggesting that their effect in vivo is not solely based on the dynamics of inhibitor-proteasome interactions. A dramatic accumulation of GFP was achieved by treatment with combinations of inhibitors at doses that did not independently block the ubiquitin/proteasome pathway (Fig. 5B). A uniform accumulation was induced by combination of 25 μM YL_3-VS with 100 μM ritonavir while only half of the cells treated with 50 μM NP-LLG-VS and 25 μM YL_3-VS showed accumulation of the reporter. Thus, by using mixture of different inhibitors this reporter system can be used to identify modifiers of the proteasome that do not affect block the ubiquitin-proteasome pathway individually. Such modifiers are expected to change the peptides generated from a protein substrate upon proteasomal degradation.

Example 6 - Use of the reporter to identify polypeptides that inhibit in trans the ubiquitin/proteasome pathway.

Recently a mutant ubiquitin protein has been discovered in affected neurons of patients with Alzheimer's disease and Down syndrome but not in neurons of healthy individuals (ref). Expression of this aberrant is caused by molecular misreading of the ubiquitin B gene resulting in mRNA transcripts that encode ubiquitin with a +1 frame-shift close to the 3' end of the ubiquitin open reading frame (ref). The resulting Ub^{+1} product consists of a wild type ubiquitin with the last amino acid mutated and an aberrant 19 amino acid C-terminal extension. Based on in vitro data it has been postulated that the Ub^{+1} molecules may competitively inhibit binding of polyubiquitinated substrates to the proteasome, which is expected to result in a general blockage of the ubiquitin/proteasome pathway (ref).

The inventors addressed the question whether the postulated inhibitory activity of Ub^{+1} polypeptide could be revealed with the usage of the GFP reporters for proteasomal degradation. This question is difficult to address using conventional methods since polypeptides that block overall proteasomal degradation are expected to induce apoptosis in the transfected cells making it difficult to generate stable Ub^{+1} transfectants. Moreover analysis of the ubiquitin/proteasome pathway in transiently

transfected cells is complicated by the presence of both transfected and untransfected cells within the population. To explore the functionality of the reporter cells, HeLa cells stably expressing the Ub^{G76V}-GFP reporter were transiently transfected with a Ub+1-encoding plasmid or with a control plasmid encoding wild type FLAG-tagged ubiquitin. Two days post-transfection the cells were stained with an anti-Ub^{+1} antibody and analyzed by fluorescence microscopy. It was observed that many of the cells expressing Ub^{+1} demonstrated a dramatic accumulation of the GFP reporter as measured by the GFP fluorescence indicating an obstruction in the ubiquitin/proteasome-dependent proteolysis in these cells (Fig 6A). Quantification of the percentage of transfected cells emitting GFP fluorescence above background levels showed that approximately 80% of the Ub^{+1}-expressing HeLa cells accumulated the GFP reporter compared to less than 5% of the cells expressing the wild type FLAG-tagged ubiquitin (Fig. 6B). This demonstrates that the reporter-expressing cells can be used to identify polypeptides that interfere with proteasomal degradation. Moreover, these cells can be used to conveniently quantify the inhibitory activity by determining the percentage of affected cells and their fluorescence intensity.

Example 7 – Use of the reporter to identify polypeptide sequences that inhibit in cis the ubiquitin/proteasome pathway.

Several studies have shown that the viral EBNA1 protein is protected from proteasomal degradation by means of a long repetitive sequence consisting of solely glycine and alanine amino acids (ref). Interestingly this glycine-alanine repeat (GAr) functions as a transferable element and when inserted into another proteasome substrates the new host protein is protected from proteasomal degradation. Moreover, the GAr works exclusively in cis and does not affect turnover of other proteasome substrates (ref). Although its mode of action is unknown, detailed in vitro and in vivo analysis revealed that substrates harbouring the GAr are efficiently ubiquitinated but fail to be processed (ref). In a set of experiments the inventors assessed the applicability of the GFP reporters in monitoring and quantifying the in cis inhibitory effect of proteasomal degradation mediated by the viral GAr. GArs of different lengths were linked to the C-terminus

of four GFP reporters varying in stability from completely stable to highly destabilized (Fig. 7A). HeLa cells were transiently transfected in duplicate with the GAr-harbouring GFP reporters and from 16 until 26 hr post-transfection the cells were either left untreated or incubated with 10 μM Z-L_3-VS proteasome inhibitor. Western blot analysis with an anti-GFP antibody revealed that insertion of a 25 amino acid GAr in the modestly destabilized Ub-P-GFP reporter resulted in increased steady state levels compared with its repeat-less counterpart (Fig. 7B). Moreover, addition of proteasome inhibitor increased the steady state levels of the Ub-P-GFP reporter while the Ub-P-GFP GA25 levels did not further increase. On the contrary, the very low steady state levels observed with Ub-R-GFP and Ub^{G76V}-GFP did not increase upon insertion of the 25 amino acid GAr (Fig. 7B). Together these data show that this small GAr protects a GFP reporter which is only weakly targeted for proteasomal degradation whereas the repeat in the context of the two highly destabilized reporters did not have a measurable effect in this particular assay.

Subsequently the effect of the short 25 and the 239 amino acid long GAr was compared by transfection and flow cytometric analysis. The inventors used the ratio between the percentage of positive cells in the absence or presence of proteasome inhibitor as a measure for proteasomal degradation. Two stable controls, GFP and Ub-M-GFP, resulted values close to 1 confirming that these proteins are not processed by the proteasome and give equal percentage of fluorescent cells in the absence or presence of the inhibitor (Fig. 7C). The destabilized Ub-R-GFP and Ub^{G76V}-GFP significantly reduced this value as expected for a true proteasome substrate. Insertion of the 25 amino acid and 239 amino acid GAr resulted in a length dependent increase of the ratio. This shows that the GAr does also protect the strongly destabilized reporters even though it does not reach a full protective effect. Thus, the GFP reporter system can be used to identify and characterize polypeptide sequences that inhibit in cis degradation of proteasome substrates.

Table I. Inhibition of enzymatic activity of proteasomes[a].

	% Inhibition		
Inhibitor	Chymotrypsin-like	Trypsin-like	PGPH
10 μM Z-L_3-VS	97.5 ± 0.4	53.3 ± 0.3	89.7 ± 1.5
50 μM NP-LLG-VS	62.6 ± 5.0	12.1 ± 2.5	22.6 ± 5.5
25 μM YL_3-VS	36.8 ± 5.9	29.3 ± 1.7	22.0 ± 10.1
50 μM NP-LLG-VS + 25 μM YL_3-VS	81.3 ± 2.9	49.0 ± 1.8	62.1 ± 4.0

a. The chymotrypsin-like, trypsin-like and PGPH activities of the partially purified proteasome from untreated cells and cells cultured for 15 h in the presence of 10 μM Z-L_3-VS, 50 μM NP-LLG-VS, 25 μM YL_3-VS and 50 μM NP-LLG-VS + 25 μM YL_3-VS were analysed by cleavage of the fluorogenic substrates Suc-LLVY-AMC, Boc-LRR-AMC and Cbz-LLE-βNA, respectively. The data are presented as percentage residual enzymatic activities of proteasomes from treated cells relative to proteasomes from untreated controls. Mean ± SD of triplicate tests. One representative experiment out of three performed with different proteasome preparations.

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Claims

1. A reporter protein construct comprising a reporter part at the carboxy terminal and an amino terminal part comprising ubiquitin (Ub) or a functional analogue of ubiquitin, that may further comprise a linker peptide between the reporter part and the ubiquitin, whereby the linker comprises at least one Lys-residue, and whereby position 3 of the linker is occupied by a Lys-residue.

2. A reporter protein construct according to claim 1, wherein the length of the linker is in the interval from 3-30 amino acids, preferably 11-17 amino acids.

3. A reporter protein construct according to claim 1 or 2, wherein the reporter part is a fluorescent protein, such as GFP.

4. A reporter protein construct according to any one of claim 1-3, wherein the amino terminal part is wild type ubiquitin or ubiquitin in which residue 76 is substituted by a non-glycine amino acid.

5. A reporter protein construct according to any one of claims 1-4, wherein the linker has the formula

$$N_1X_2K_3X_{8\text{-}10}$$

where N_1 is Arg, Glu, Gln, Trp, Phe, Lys, His, Tyr, Asp, Ala, Ser, Leu, Thr, Val, Ile, Gly or Cys, preferably Arg, Glu, Gln, Trp, Phe, Lys, His, Tyr or Asp;
X_2 is any residue;
K_3 is a Lys residue;
$X_{8\text{-}10}$ is a polypeptide of 8, 9 or 10 amino acid residues of any type.

6. A reporter protein construct according to claim 5, having the formula

$$N_1X_2K_3(X(K))_{8\text{-}10}$$

where $(X(K))_{8\text{-}10}$ is a polypeptide of 8,9 or 10 amino acids, with one or more Lys residues in any position(s).

7. A reporter protein construct according to any of claims 1-4, having the formula

$$N_1X_2K_3X_{11\text{-}13}K_c$$

where N_1 is Arg, Glu, Gln, Trp, Phe, Lys, His, Tyr, Asp, Ala, Ser, Leu, Thr, Val, Ile, Gly or Cys, preferably Arg, Glu, Gln, Trp, Phe, Lys, His, Tyr or Asp;

X_2 is any residue;

K_3 is a Lys residue;

$X_{11\text{-}13}$ is a polypeptide of 11, 12 or 13 amino acids,

K_c is a Lys residue in position 15, 16 or 17.

8. A reporter protein construct according to claim 7, having the formula

$$N_1X_2K_3(X(K))_{11\text{-}13}K_c$$

where $(X(K))_{11\text{-}13}$ is a polypeptide of 11, 12 or 13 amino acids, with one or more Lys residues in any position(s).

9. A reporter protein construct according to any one of the preceding claims, wherein the linker has the sequence:

XGKLGRQDPPVAT (SEQ.ID.NO.1),

wherein X is M, R, L, P, or V.

10. A nucleic acid construct, such as a DNA or an RNA construct, encoding the reporter protein construct according to any one of claims 1-9.

11. A cell line, such as HeLa or any equivalent cell, that stably expresses the reporter constructs of claims 1-9, and that can be used for monitoring the accumulation of the reporter in living cells.

12. A vector expressing a reporter construct according to any one of claims 1-9.

13. A method for quantification of the activity of the ubiquitin/proteasome pathway, characterised by that the method is performed in living cells, and that it comprises the steps of (1) expressing a reporter construct of any one of claims 1-9 into the cell, (2) optionally adding substances to inhibit ubiquitin/proteasome

dependent proteolysis, and (3) measuring the amount of accumulated reporter protein.

14. A method according to claim 13, wherein the method is for predicting cell death by inhibition of the ubiquitin/proteasome pathway.

15. A method according to claim 13, wherein the method is for identification of compounds that selectively modify the enzymatic activity of the proteasome without causing cell death.

16. A method according to claim 13, wherein the method is for assessing the endogenous level of activity of the ubiquitin-proteasome pathway.

17. A method according to claim 13, wherein the method is for screening for substances that modify different steps of the ubiquitin pathway, comprising ubiquitin activation, ubiquitin ligation, ubiquitin transcription/conjugation, recognition of the ubiquitin substrates by the proteasome, or clearing of the ubiquitin adducts by ubiquitin hydrolases.

18. Use of a DNA construct according to claim 10 as a reporter of transcriptional activation of a promoter of interest, comprising the following steps:

a. cloning of the DNA downstream of the promoter of interest in a plasmid;
b. expression of the reporter plasmid in a cell;
c. optionally adding substances that modify the activity of the promoter;
d. measuring the presence of accumulated reporter protein.

19. A linker peptide having a reporter protein part at its carboxy terminal end, and a ubiquitin part at its amino terminal end, having the general formula:

$N_1X_2K_3(X_b)_n$

where N is Arg, Glu, Gln, Trp, Phe, Lys, His, Tyr, Asp, Ala, Ser, Leu, Thr, Val, Ile, Gly or Cys, preferably Arg, Glu, Gln, Trp, Phe, Lys, His, Tyr or Asp;

X is any residue;

K is a Lys residue;

$(X_b)_n$ is a polypeptide of 8, 9 or 10 amino acid residues of any type.

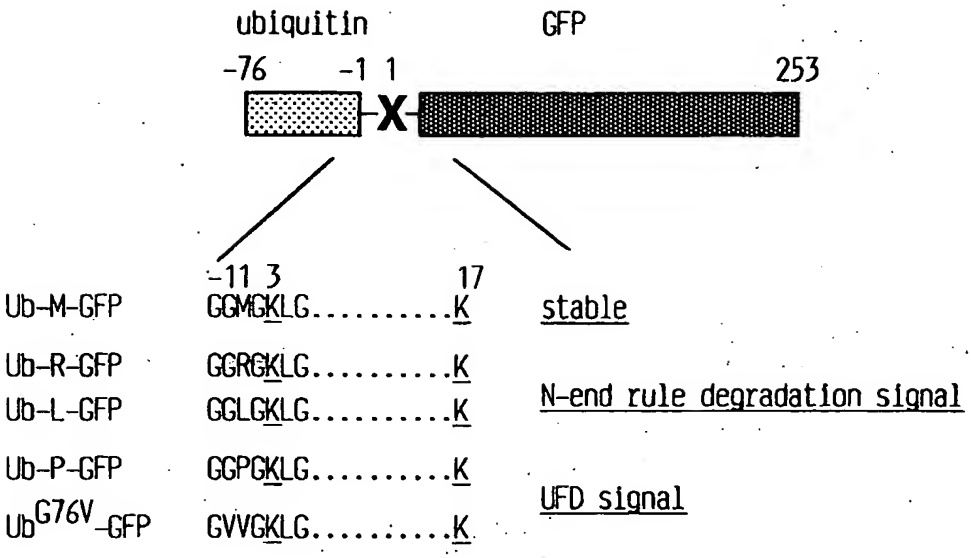

ubiquitin
GFP
-76
-1 1
253
X
-11 3
17
Ub-M-GFP GGMGKLG.....K stable
Ub-R-GFP GGRGKLG.....K
Ub-L-GFP GGLGKLG.....K N-end rule degradation signal
Ub-P-GFP GGPGKLG.....K
UbG76V-GFP GVVGKLG.....K UFD signal

FIG. 1A

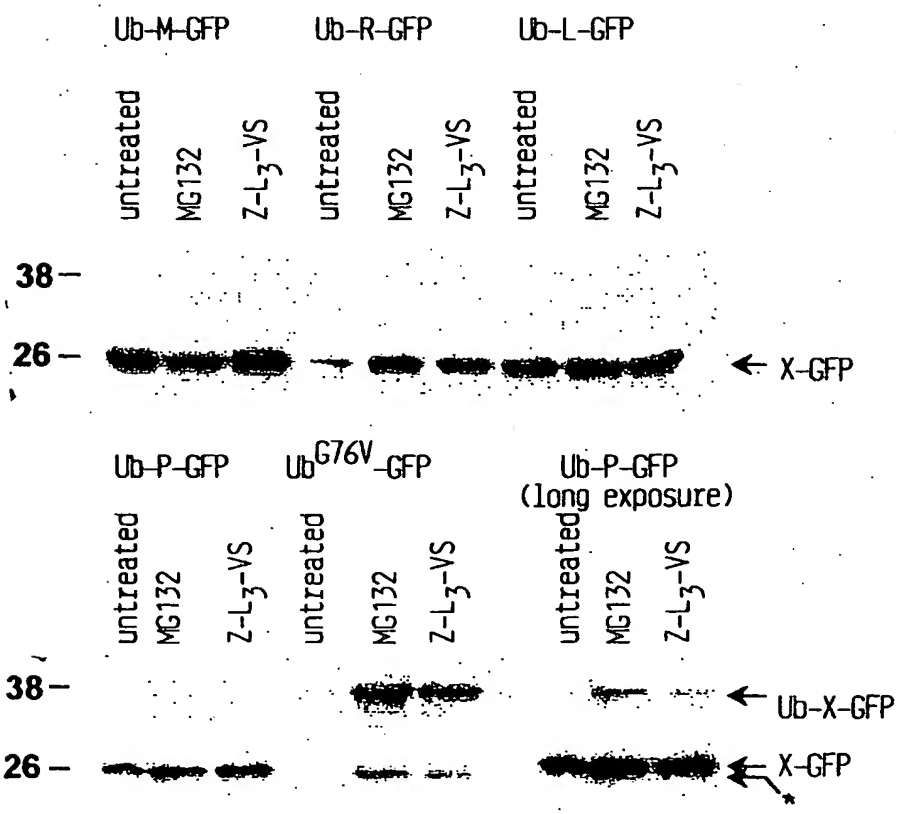

Ub-M-GFP
Ub-R-GFP
Ub-L-GFP
untreated
MG132
Z-L3-VS
38
26
X-GFP
Ub-P-GFP
UbG76V-GFP
Ub-P-GFP (long exposure)
Ub-X-GFP
X-GFP

FIG. 1B

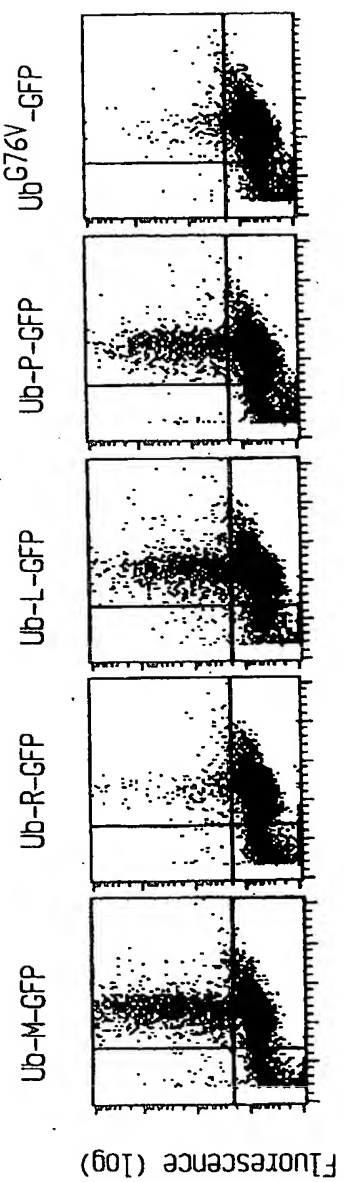

Ub-M-GFP
Ub-R-GFP
Ub-L-GFP
Ub-P-GFP
Ub^{G76V}-GFP
Fluorescence (log)
Positive: 13%
Mean FL: 949
Positive: 2%
Mean FL: 457
Positive: 10%
Mean FL: 439
Positive: 8%
Mean FL: 447
Positive: 2%
Mean FL: 238

FIG.2A

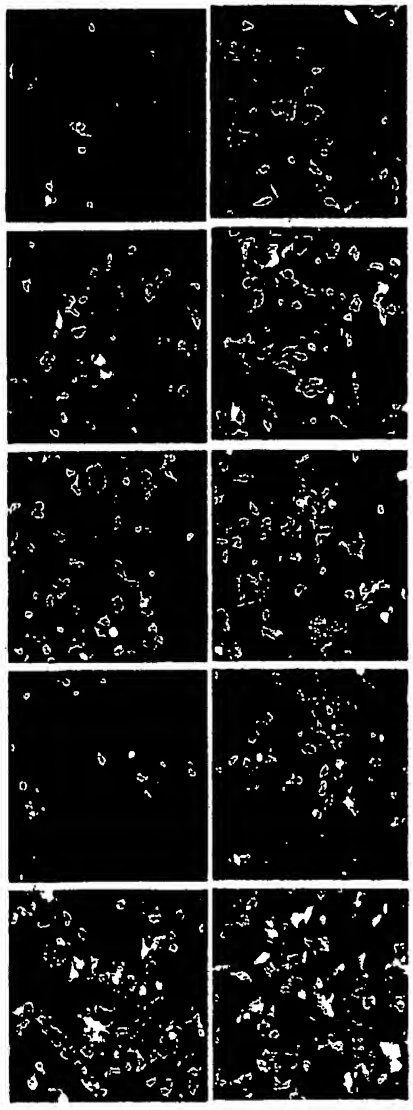

untreated
MG132

FIG.2B

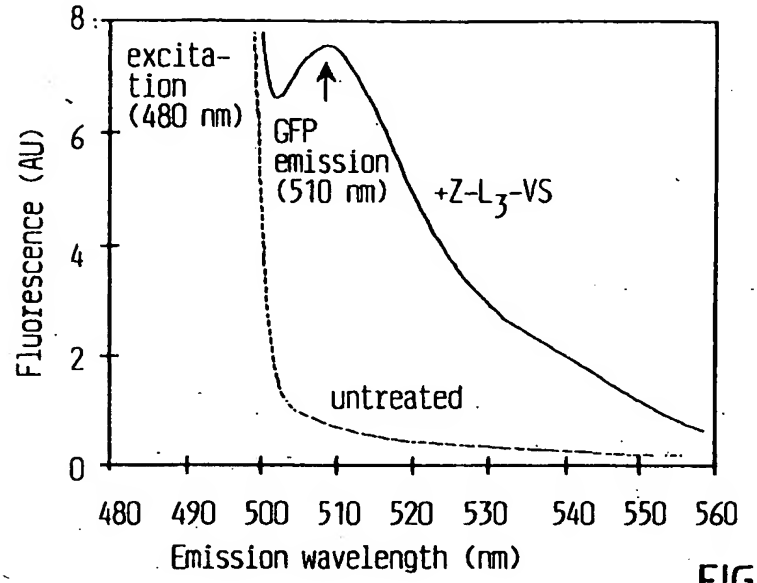

8
6
4
2
0
Fluorescence (AU)
excita-
tion
(480 nm)
GFP
emission
(510 nm)
+Z-L3-VS
untreated
480 490 500 510 520 530 540 550 560
Emission wavelength (nm)

FIG.3A

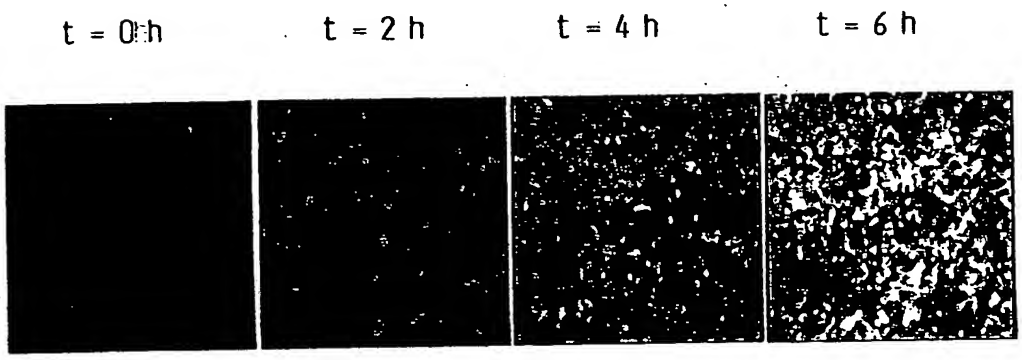

t = 0 h
t = 2 h
t = 4 h
t = 6 h

FIG.3B

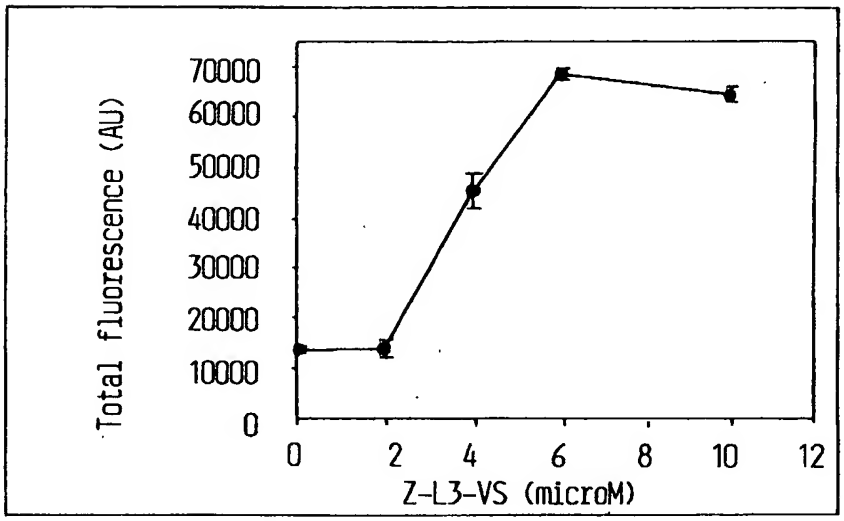

Total fluorescence (AU)
70000
60000
50000
40000
30000
20000
10000
0
0
2
4
6
8
10
12
Z-L3-VS (microM)

FIG.3C

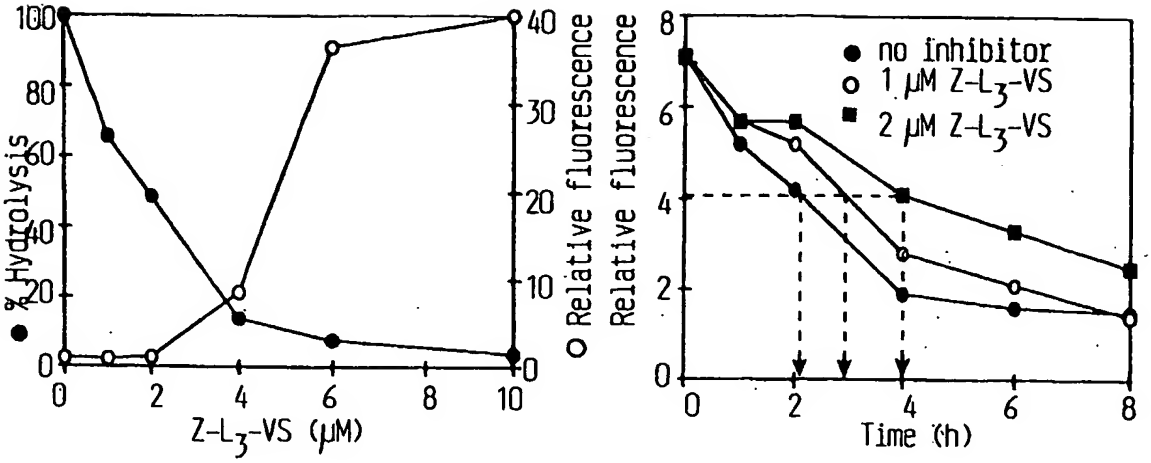

% Hydrolysis
Relative fluorescence
Z-L3-VS (μM)
no inhibitor
1 μM Z-L3-VS
2 μM Z-L3-VS
Time (h)

FIG.4A

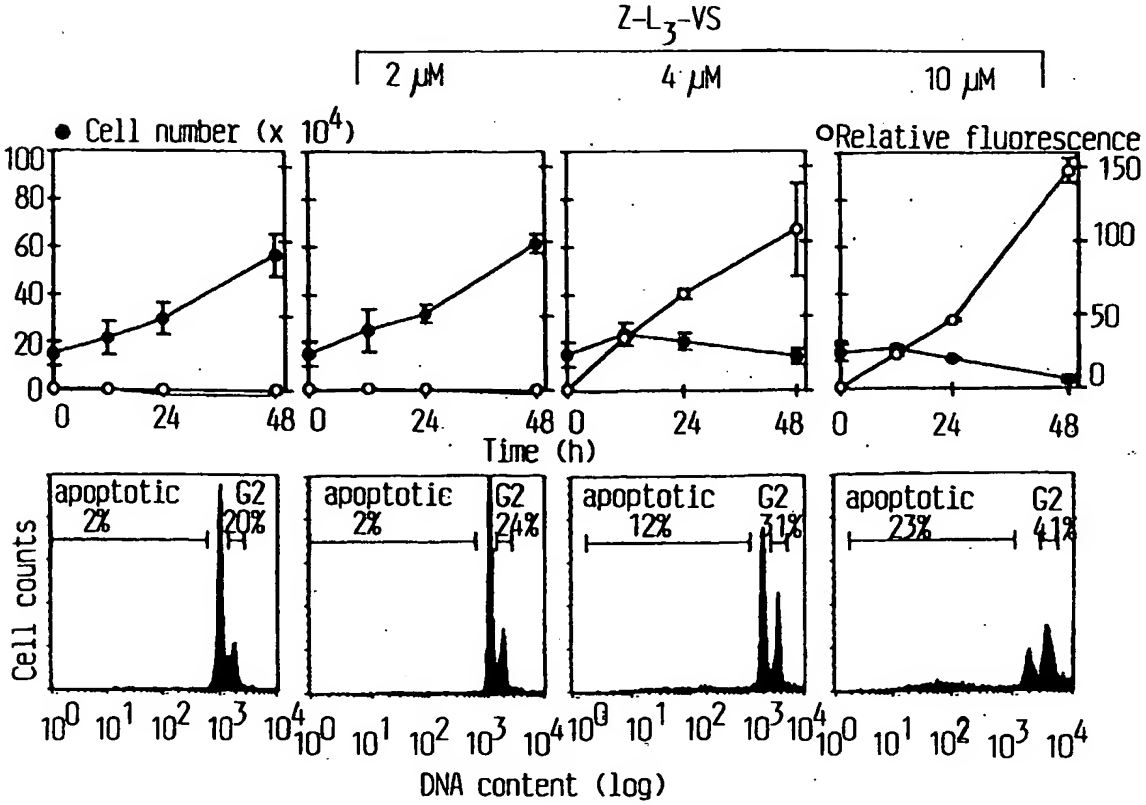

Z-L3-VS
2 μM
4 μM
10 μM
Cell number (x 10^4)
Relative fluorescence
Time (h)
apoptotic 2%
G2 20%
apoptotic 2%
G2 24%
apoptotic 12%
G2 31%
apoptotic 23%
G2 41%
Cell counts
DNA content (log)

FIG.4B

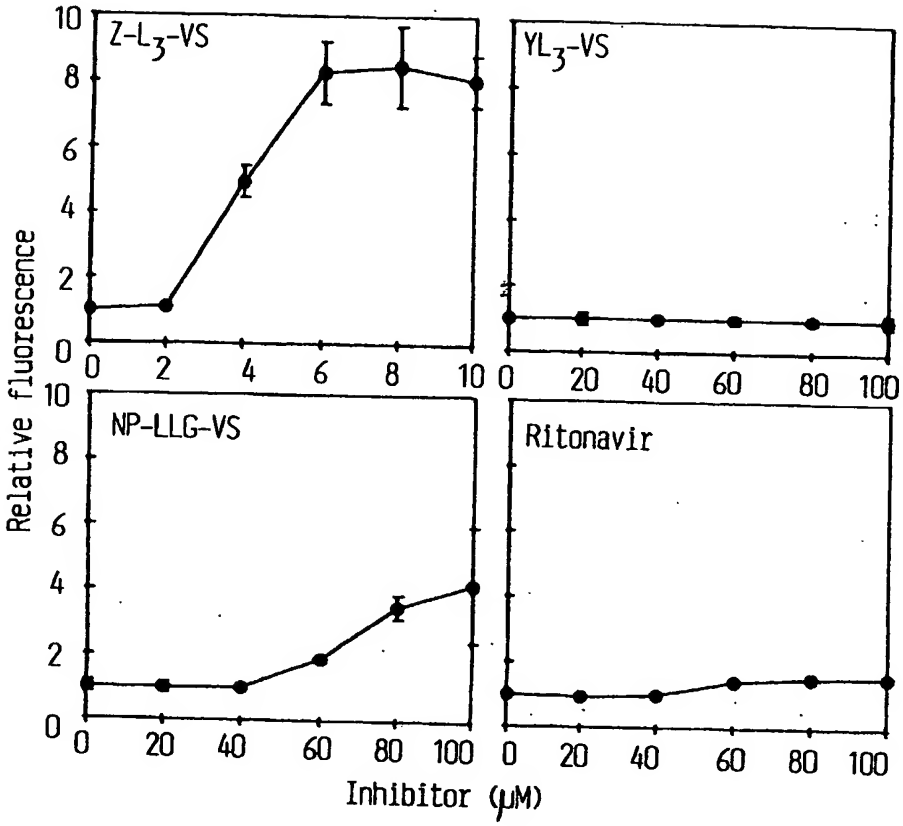

Z-L3-VS
YL3-VS
NP-LLG-VS
Ritonavir
Relative fluorescence
Inhibitor (μM)
0
2
4
6
8
10
20
40
60
80
100

FIG.5A

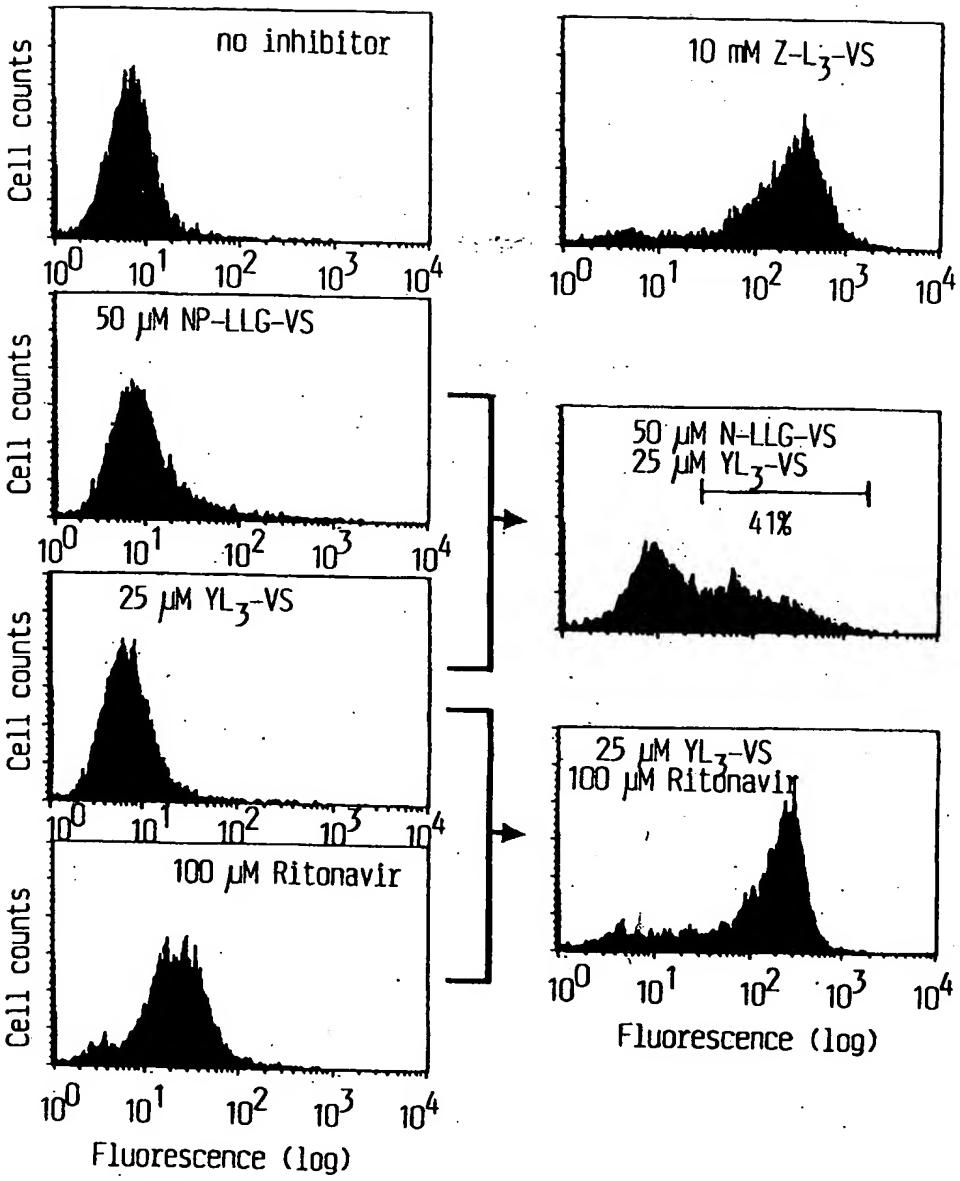

no inhibitor
10 mM Z-L3-VS
50 µM NP-LLG-VS
50 µM N-LLG-VS
25 µM YL3-VS
41%
25 µM YL3-VS
25 µM YL3-VS
100 µM Ritonavir
100 µM Ritonavir
Cell counts
Fluorescence (log)

FIG. 5B

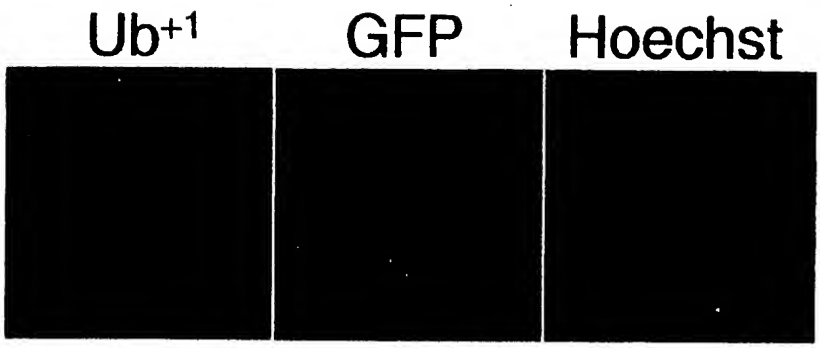

Ub+1
GFP
Hoechst

FIG.6A

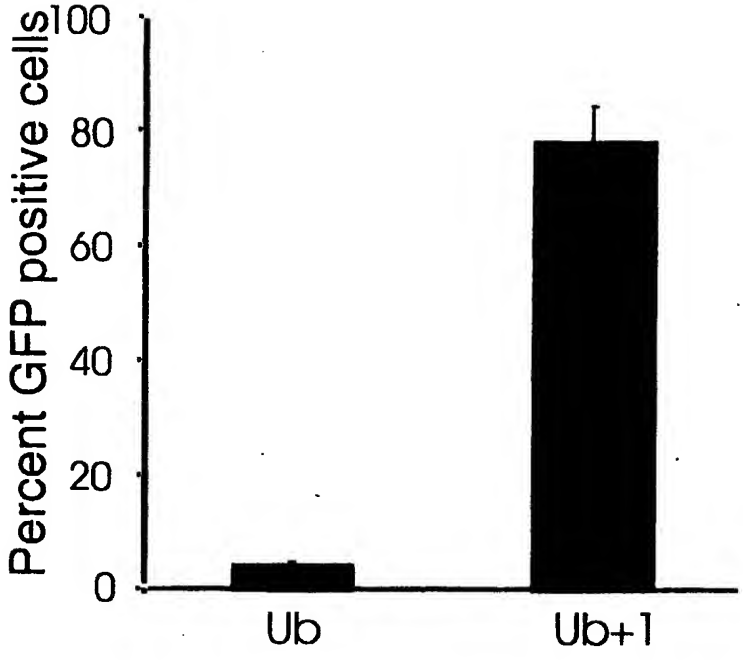

Percent GFP positive cells
100
80
60
40
20
0
Ub
Ub+1

FIG.6B

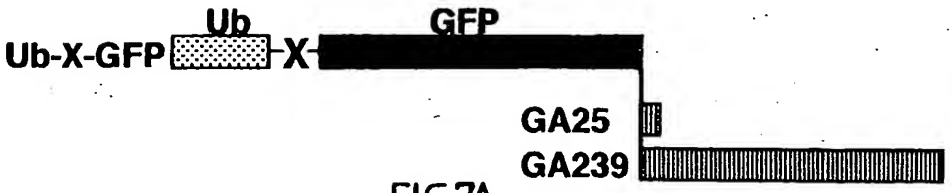
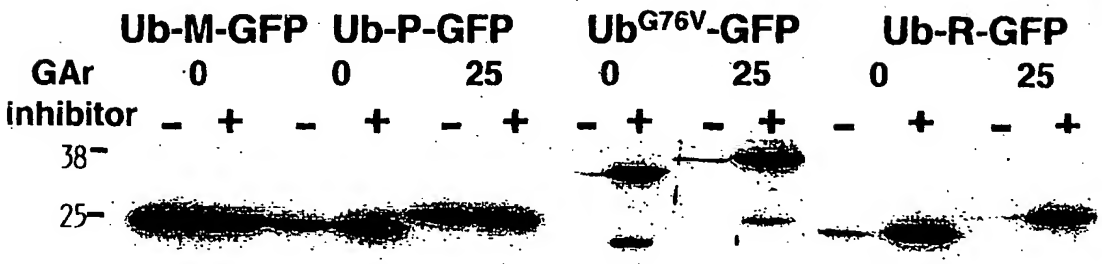


FIG.7A



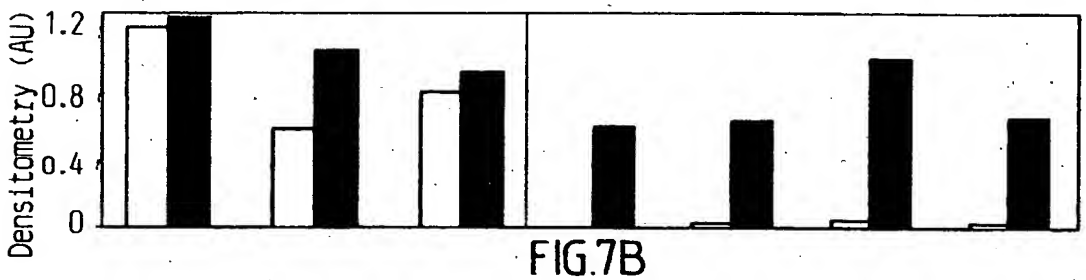


FIG.7B

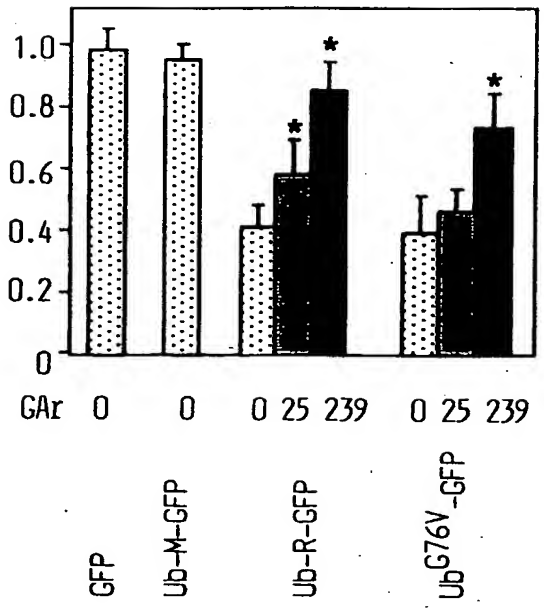


FIG.7C

SEQUENCE LISTING

<110> ACCURO IMMUNOLOGY AB

<120> REPORTER PROTEIN CONSTRUCT

<130> PROTEASOMHÄMMARE

<140>
<141>

<160> 1

<170> PatentIn Ver. 2.1

<210> 1
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Xaa(1) = Met, Arg, Leu, Pro, or Val

<220>
<223> Description of Artificial Sequence:PEPTIDE

<400> 1
Xaa Gly Lys Leu Gly Arg Gln Asp Pro Pro Val Ala Thr
1 5 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 01/00921

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07K 19/00, C12N 15/09
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CHEM.ABS. EPO INTERNAL, WPI DATA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	NatureBiotechnology, Volume 18, May 2000, Nico P. Dantuma et al, "Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells" page 538 - page 543	1-19
A	US 5132213 A (ANDREAS BACHMAIR ET AL), 21 July 1992 (21.07.92)	1-19
A	WO 9911774 A1 (FLUORESCIENCE LIMITED), 11 March 1999 (11.03.99)	1-19

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 Sept 2001	14 -09- 2001
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Gómez Lagerlöf/EÖ Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/08/01

International application No.
PCT/SE 01/00921

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5132213 A	21/07/92	CA 1339842 A	28/04/98
		EP 0408661 A	23/01/91
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		JP 3503606 T	15/08/91
		US 5093242 A	03/03/92
		US 5196321 A	23/03/93
		US 5496721 A	05/03/96
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		US 5847097 A	08/12/98
		WO 8909829 A	19/10/89
		CA 1313831 A	23/02/93
		EP 0324789 A	26/07/89
		JP 2501618 T	07/06/90
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		JP 2776414 B	16/07/98
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		JP 3038193 B	08/05/00
		JP 8252096 A	01/10/96
		JP 10057061 A	03/03/98
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		WO 8802406 A	07/04/88
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		NO 20000901 A	28/04/00